BD

Helping all people live healthy lives

Complete, Novel Reagent and Instrumentation Solutions for the Sorting of Viable, Functional Regulatory T Cells

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23-11931-00

Overview

- Introduction to regulatory T cells (Tregs)
- Reagents
  - Individual antibodies: RUO, cGMP
  - Cocktails
  - BD Pharmingen™ Human Regulatory Cell Sorting Kit
  - BD FastImmune™ Human Regulatory T Cell Function Kit
- Instrumentation
  - BD FACSAria™ flow cytometers
  - BD Influx™ systems
- Practical tips
  - Setting expectations
  - Setting up the BD FACSAria II and BD FACSAria III
  - Handling cells
  - Maximizing Treg viability
  - Performing post-sort analysis
  - Performing post-sort culture

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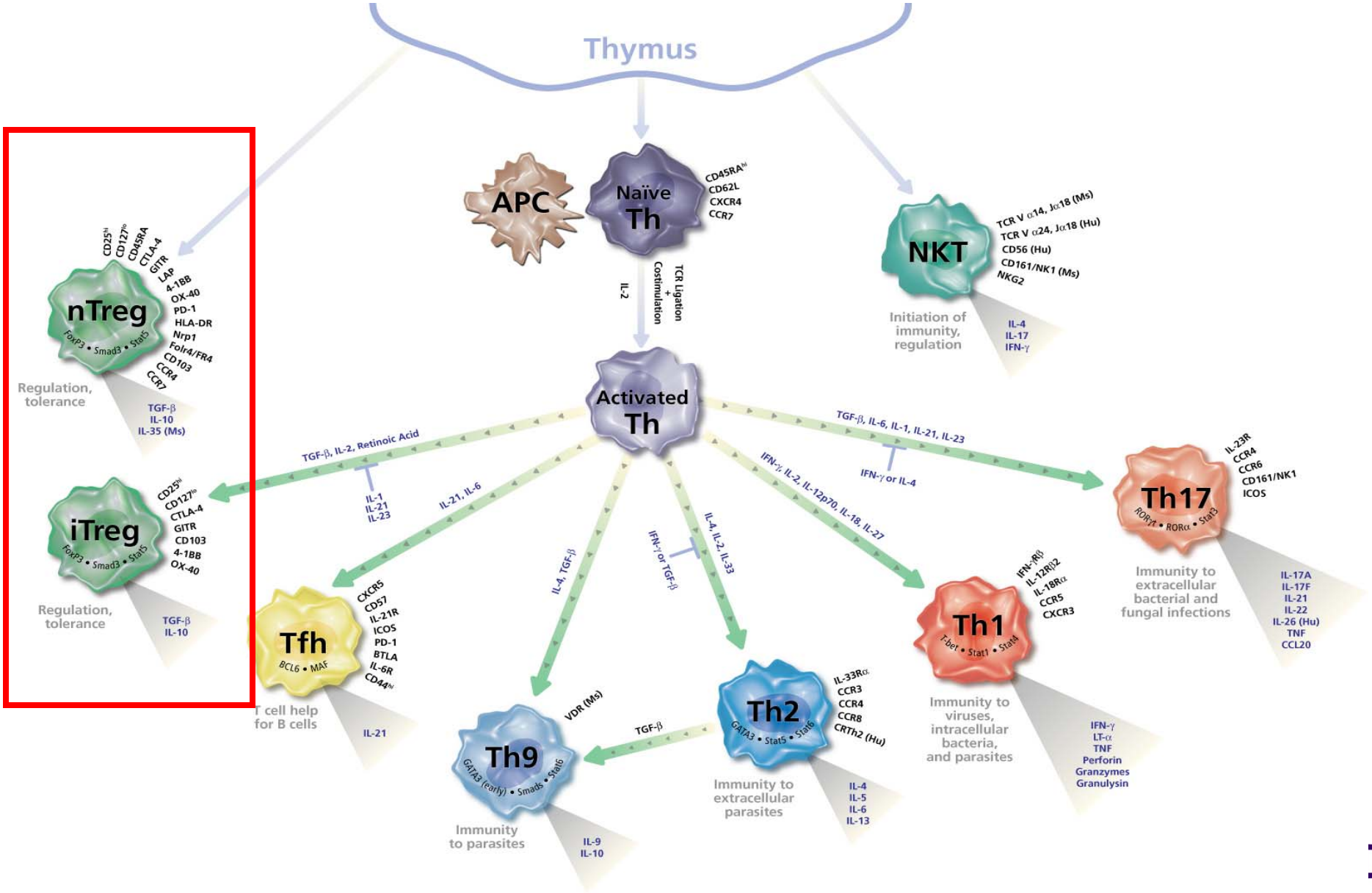
Regulatory T Cells

- Represent approximately 5–10% of $CD4^+$ cells in peripheral blood
- Play a key role in immune regulation
  - Suppress inappropriate immune responses
- Are implicated in many diseases
  - Type I diabetes
  - Graft versus host disease (GvHD)
  - Systemic lupus erythematosus
  - Rheumatoid arthritis

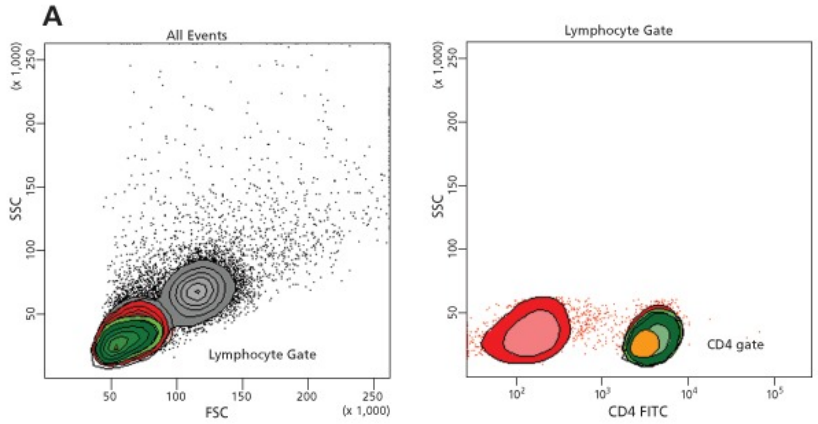
CD4+ Regulatory T Cells



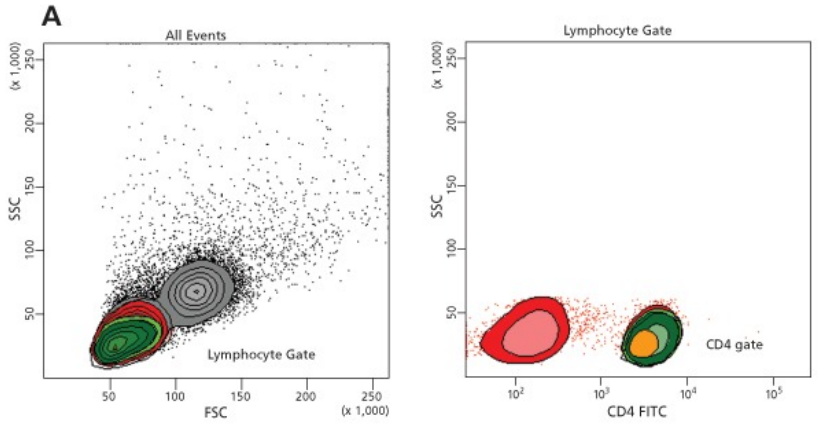
Regulatory T Cells

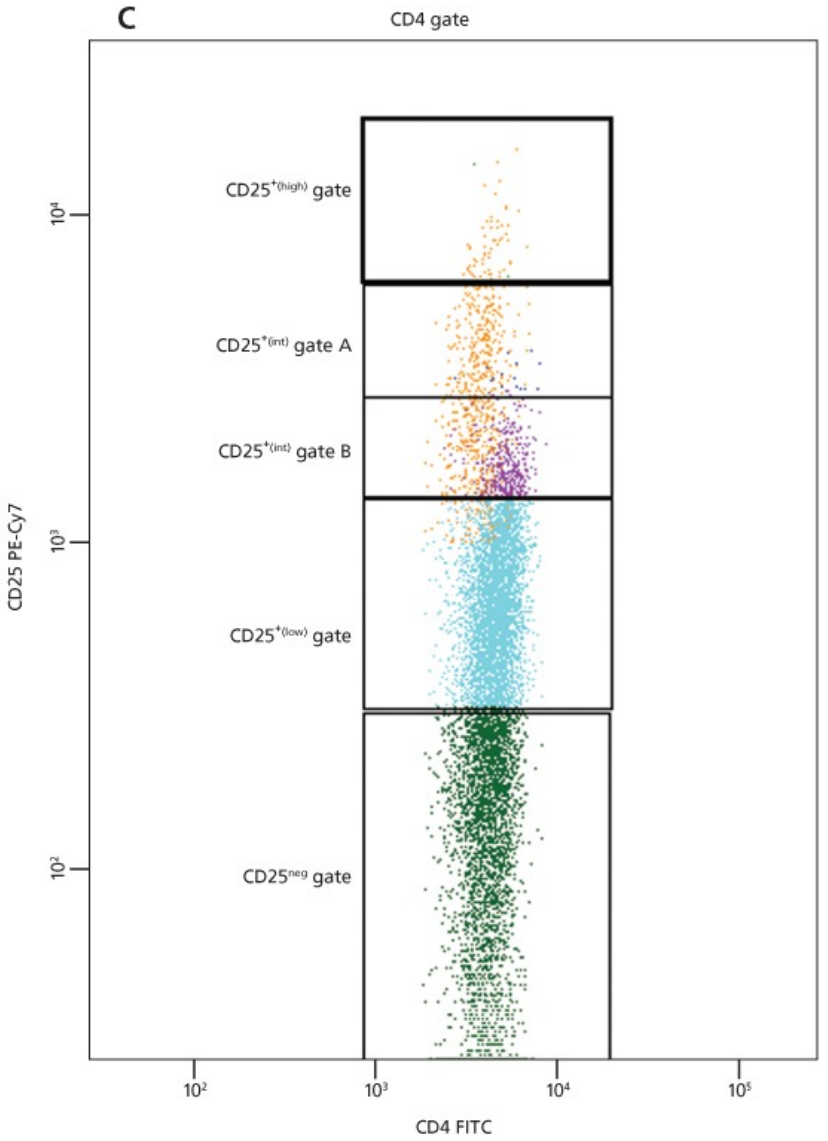
- FoxP3 is considered to be the master transcription factor for Tregs
  - Treg specific
  - Intracellular
    - Not suitable for sorting
  - Transcriptional repression of
    - IL-2
    - CD127 (IL-7 receptor)

$CD4^{+}CD25^{hi}$ Gating Strategy

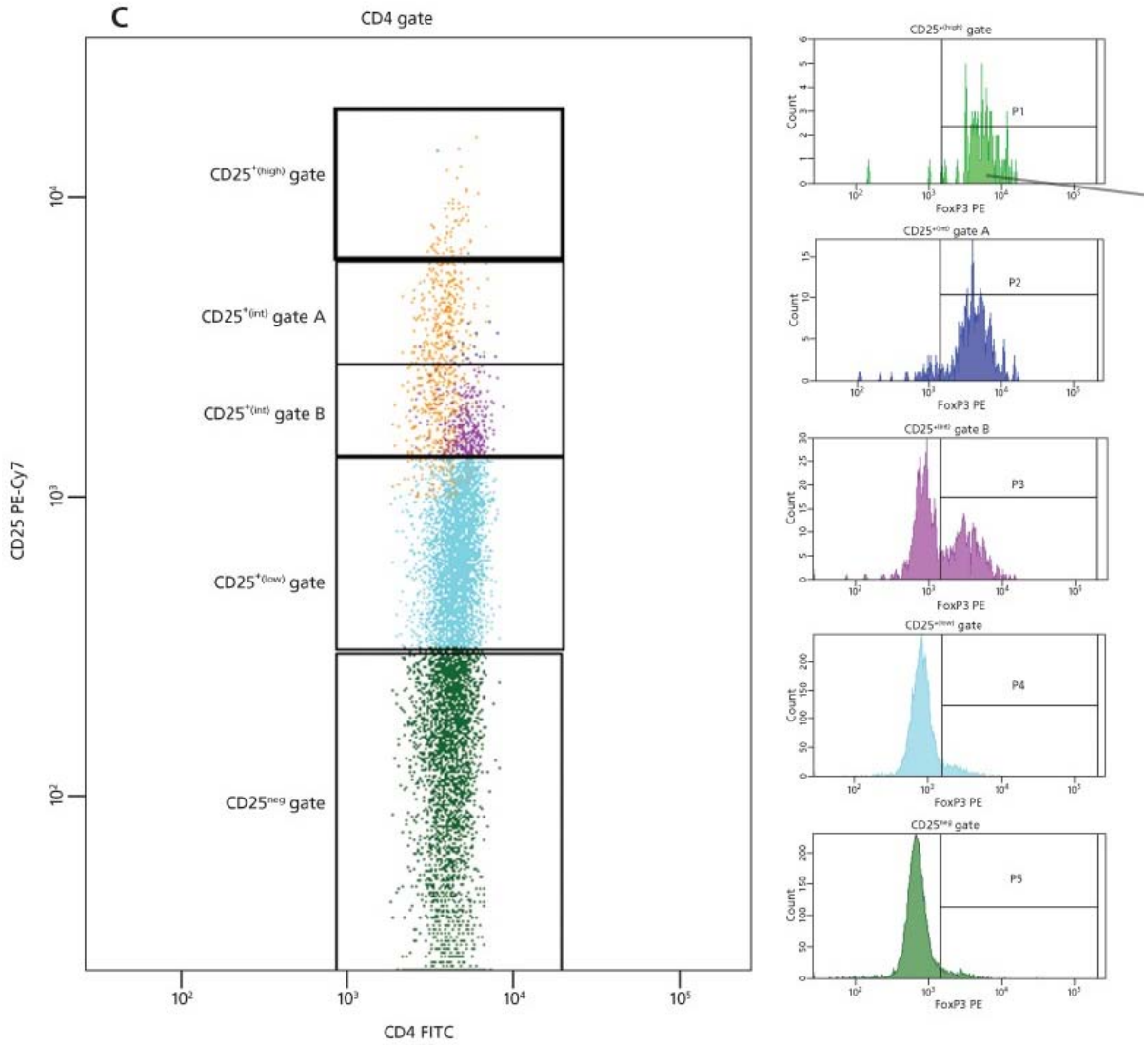


$CD4^{+}CD25^{hi}$ Gating Strategy, continued



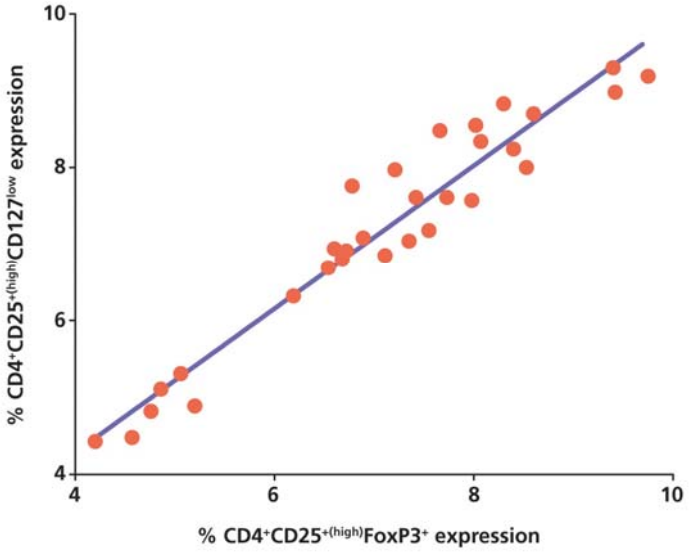


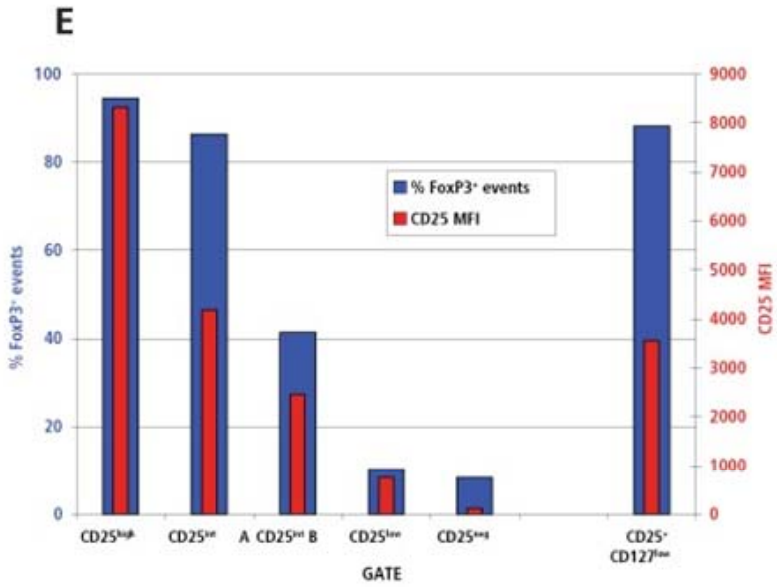
FoxP3 Expression over a Range of CD25 Levels



Regulatory T Cells

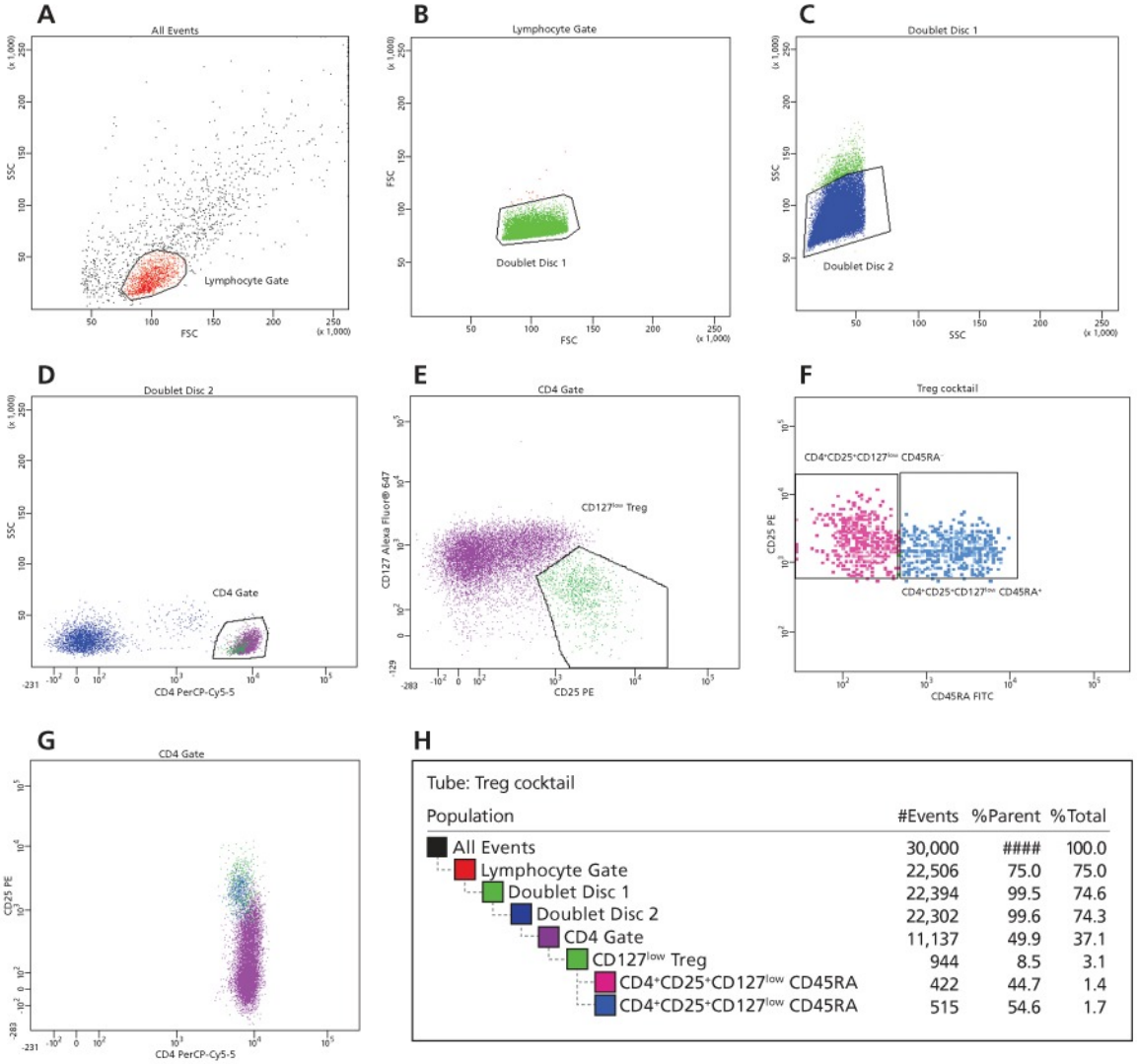
- CD127 expression inversely correlates with FoxP3.
- Use of a $CD4^+CD25^+CD127^{lo}$ gating strategy for sorting is an alternative to using $CD4^+CD25^{hi}$ alone.





BD Pharmingen Human Regulatory Cell Sorting Kit

Gating strategy for sorting



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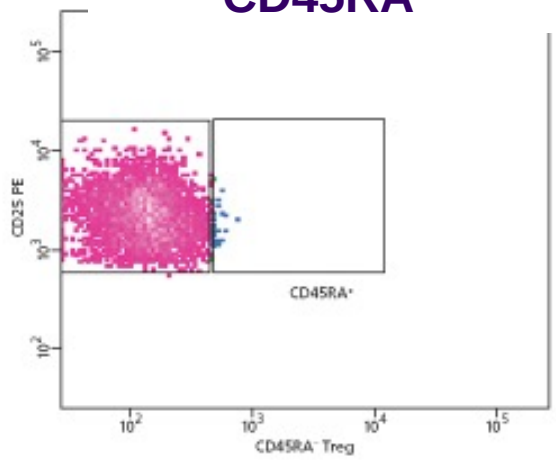
Tube: Treg cocktail

Population	#Events	%Parent	%Total
All Events	30,000	####	100.0
Lymphocyte Gate	22,506	75.0	75.0
Doublet Disc 1	22,394	99.5	74.6
Doublet Disc 2	22,302	99.6	74.3
CD4 Gate	11,137	49.9	37.1
$CD127^{low}$ Treg	944	8.5	3.1
$CD4^+CD25^+CD127^{low}$ CD45RA	422	44.7	1.4
$CD4^+CD25^+CD127^{low}$ CD45RA	515	54.6	1.7

Regulatory T Cells CD45RA Subsets

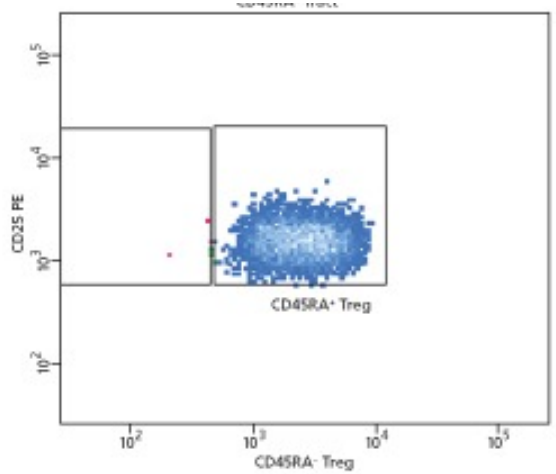
CD45RA+



Tube: CD45RAneg fract

Population	#Events	%Parent	%Total
All Events	5,502	####	100.0
Lymphocyte Gate	5,172	94.0	94.0
Doublet Disc 1	5,156	99.7	93.7
Doublet Disc 2	5,150	99.9	93.6
CD4 Gate	5,127	99.6	93.2
CD25+CD127low Treg	4,997	97.5	90.8
CD45RA- Treg	4,962	99.3	90.2
CD45RA+ Treg	23	0.5	0.4

CD45RA-



Tube: CD45RA+ fract

Population	#Events	%Parent	%Total
All Events	5,224	####	100.0
Lymphocyte Gate	4,912	94.0	94.0
Doublet Disc 1	4,898	99.7	93.8
Doublet Disc 2	4,898	100.0	93.8
CD4 Gate	4,890	99.8	93.6
CD25+CD127low Treg	4,840	99.0	92.6
CD45RA- Treg	3	0.1	0.1
CD45RA+ Treg	4,834	99.9	92.5

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Human Treg Reagents

- Individual antibodies
  - RUO
  - Special order cell processing (SOCP)
    - cGMP-produced CD4, CD25, and CD127 for specialized applications in clinical research studies
    - Same clones as in Human Regulatory T Cell Sorting Kit
- Kits
  - Human Regulatory T Cell Sorting Kit
  - BD FastImmune Human Regulatory T Cell Function Kit
  - Human Th17/Treg Phenotyping Kit
  - FoxP3 Staining Kits
- Cocktails
  - Human Regulatory T Cell Cocktail
- Human FoxP3 Buffer Set

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BD Pharmingen Human Regulatory Cell Sorting Kit

- Optimized antibody cocktail for staining
- Aliquots of individual reagents for compensation setup
  - CD4 PerCP-Cy™5.5 (Clone L200)
  - CD25 PE (Clone 2A3)
  - CD127 Alexa Fluor® 647 (Clone 40131.111)
  - CD45RA FITC (Clone HI100)
- Staining procedure
- Gating strategy for sorting
- Additional details provided in Treg application note

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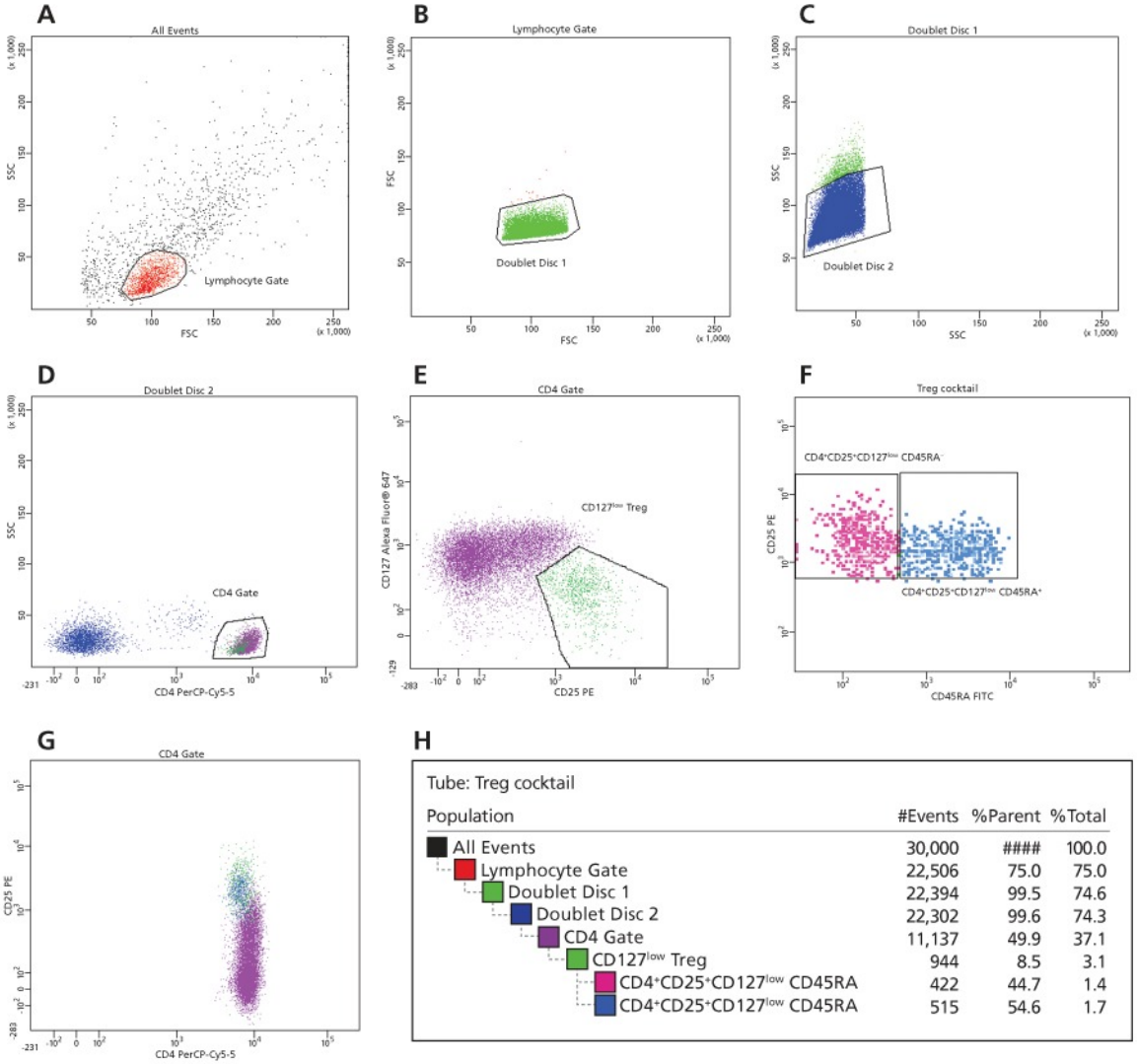
BD Pharmingen Human Regulatory Cell Sorting Kit

Gating strategy for sorting



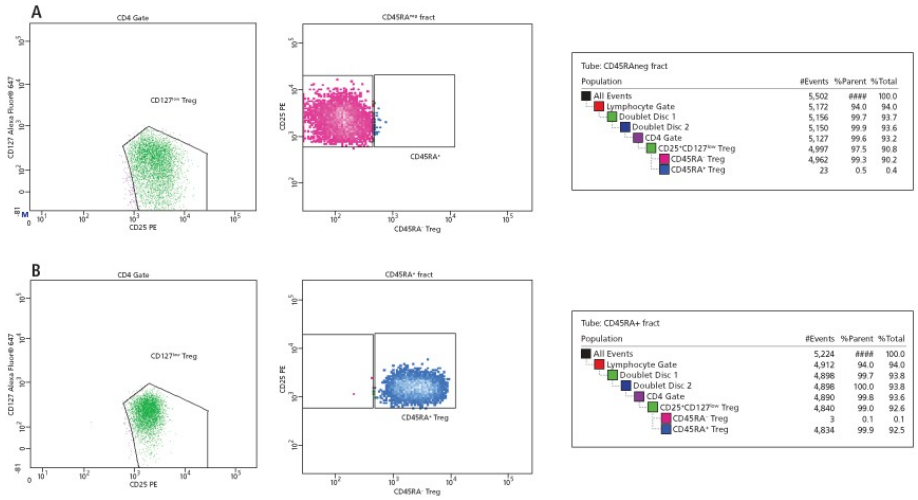
Use of nested compound gating strategy to eliminate cell aggregates and increase resolution and precision

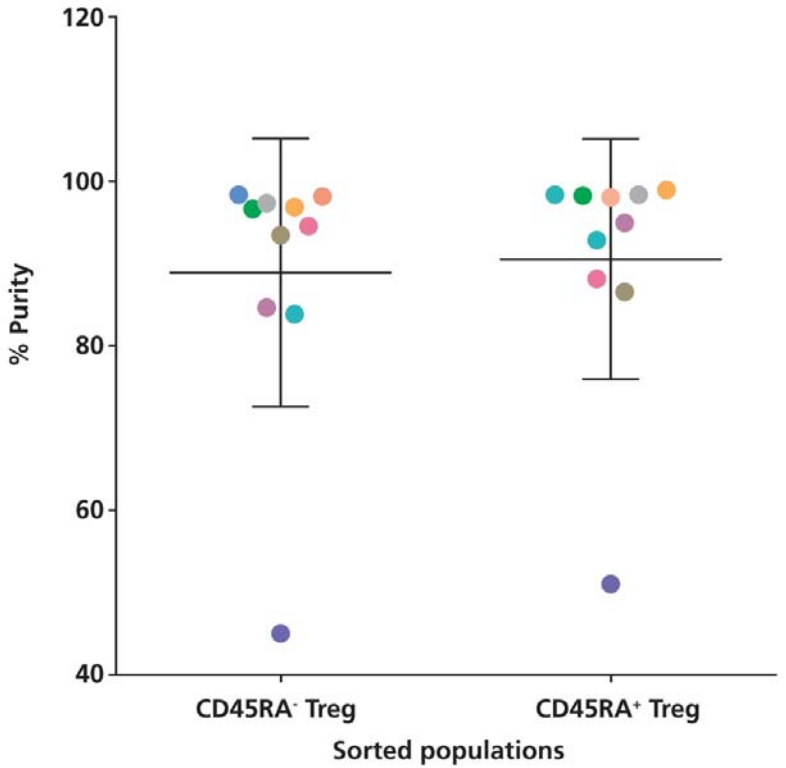
BD Pharmingen Human Regulatory Cell Sorting Kit

- Sorting Tregs on the BD FACSAria II
  - Instrument setup
    - 70-µm nozzle, 70 psi, 87 kHz
    - 100-µm nozzle, 35 psi, 60 kHz
  - Purity mode
  - 10,000–11,000 events/s
  - 12 x 75-mm serum-coated tubes
    - X-Vivo™ 15 medium + 10% Human AB serum

BD Pharmingen Human Regulatory Cell Sorting Kit

- Purity





90.6 ±14.6% CD45RA^{+} Treg (n=10)
89.4 ±14.8% CD45RA^{-} Treg (n=10)

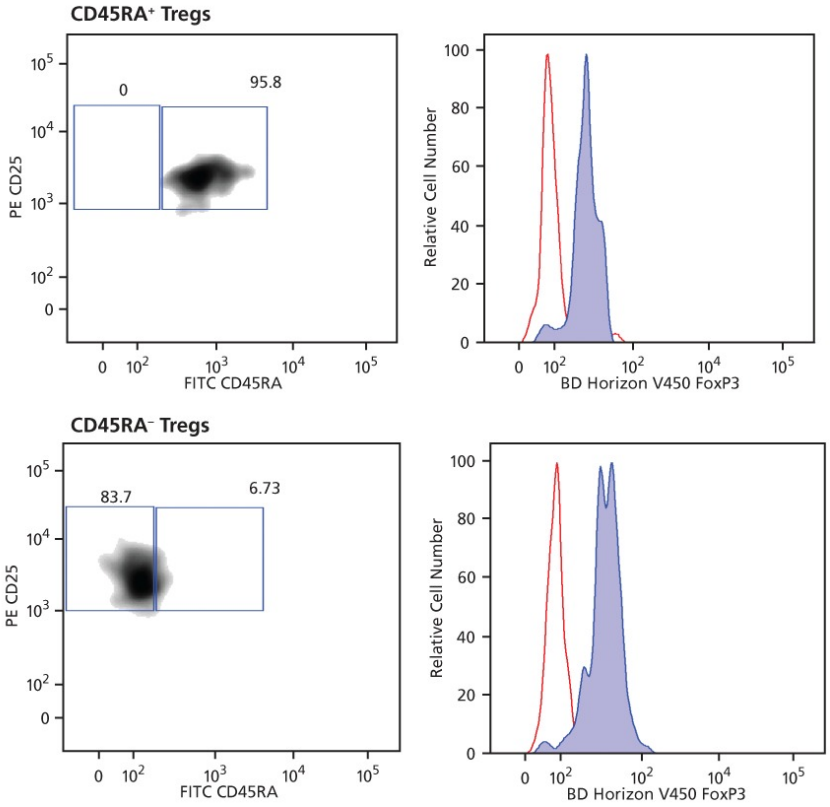
% purity = the percentage of CD4^{+}CD25^{+}CD127^{low} lymphocytes that are CD45RA^{+} or CD45RA^{-}

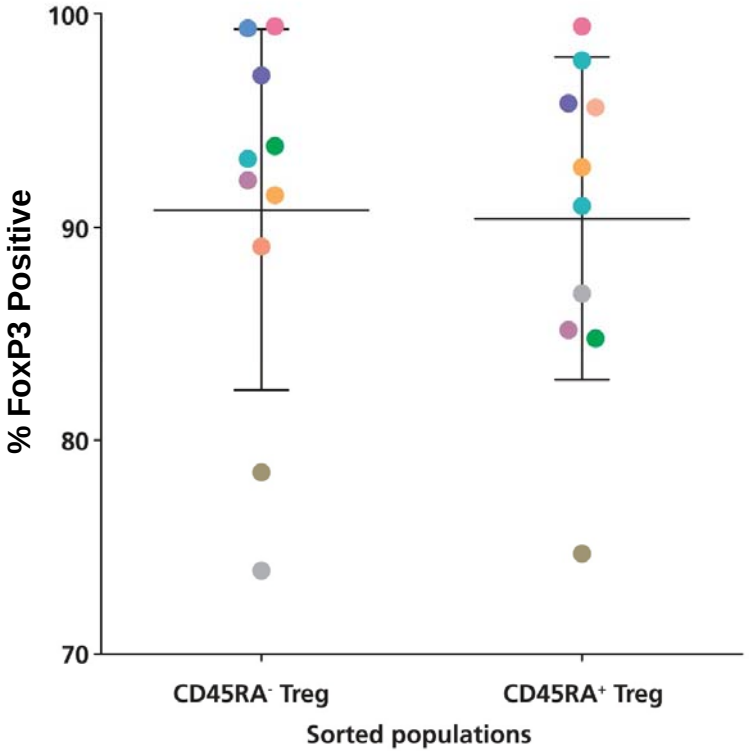
BD Pharmingen Human Regulatory Cell Sorting Kit

- FoxP3 microassay
  - Approximately 3 x 10^4 sorted cells in microcentrifuge tubes
  - Fixed and permeabilized using the FoxP3 buffer set
  - Stained with FoxP3 BD Horizon™ V450

BD Pharmingen Human Regulatory Cell Sorting Kit

FoxP3 expression





90.4 ±2.39% FoxP3$^+$ CD45RA$^+$ Treg (n=10)
90.8 ±2.67% FoxP3$^+$ CD45RA$^-$ Treg (n=10)

BD Pharmingen Human Regulatory Cell Sorting Kit

- Viability
  - Pre-sort
    - 96.4 ±1.8% (n=10)
  - Post-sort
    - $CD45RA^+$ 84.4 ±2.2% (n=3)
    - $CD45RA^-$ 85.6 ±1.5% (n=3)
- Recovery
  - Range
    - 45–100% recovery (n=10)

Statistic	Total cell number (x 10^6)		
	PBMCs sorted	**$CD45RA^-$ Tregs recovered**	**$CD45RA^+$ Tregs recovered**
Mean	77.300	0.217	0.120
1 SD	23.099	0.153	0.081
N	10	10	10

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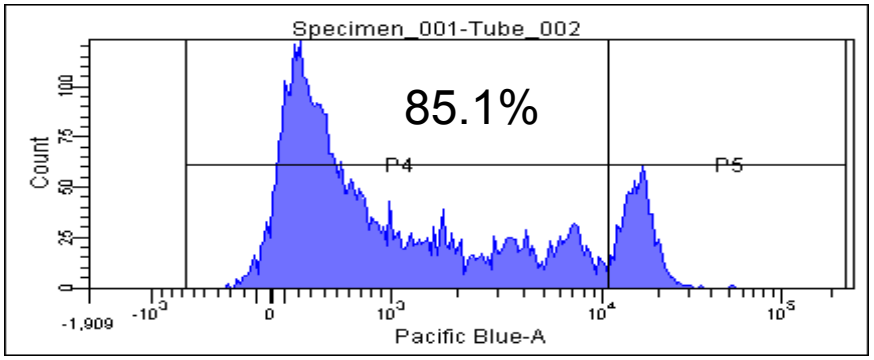
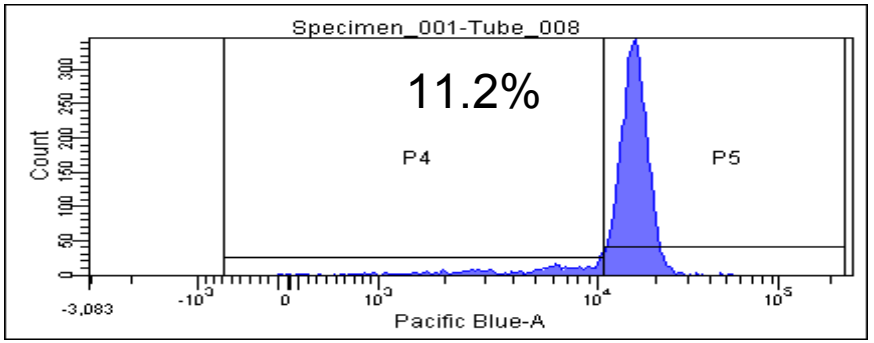
BD Pharmingen Human Regulatory Cell Sorting Kit

- Post-sort culture of $CD45RA^{+}$Tregs
  - 4 donors
  - 13–14 days
  - 2 x 10^5 cells/mL in X-Vivo15 medium + 10% AB serum
    - CD3/CD28 bead stimulation
      - Day 0 and day 9
    - 100 ng/mL of rapamycin
      - Withdrawn on day 7
    - 300 units/mL of IL-2
      - From day 2

Treg Function

- Conventional suppression assay
  - Five-day proliferation assay
    - Time consuming
    - Difficult to reproduce
    - May produce false-positive results
      - Depletion of IL-2
      - Apoptosis of rapidly dividing cells





BD FastImmune Human Regulatory T Cell Function Kit

- Measures expression of activation markers on effector T cells
  - CD154
  - CD69
- Reduced expression in the presence of Tregs
- 96-well plate format
- Short term: 7-hour activation

BD FastImmune Human Regulatory T Cell Function Kit

- Optimized antibody cocktail for staining
  - CD4 FITC
  - CD25 PE
  - CD3 PerCP-Cy5.5
- Activation markers
  - CD154 APC
  - CD69 PE-Cy™7
- Detailed assay and staining procedures
- Gating strategy for analysis

BD FastImmune Human Regulatory T Cell Function Kit

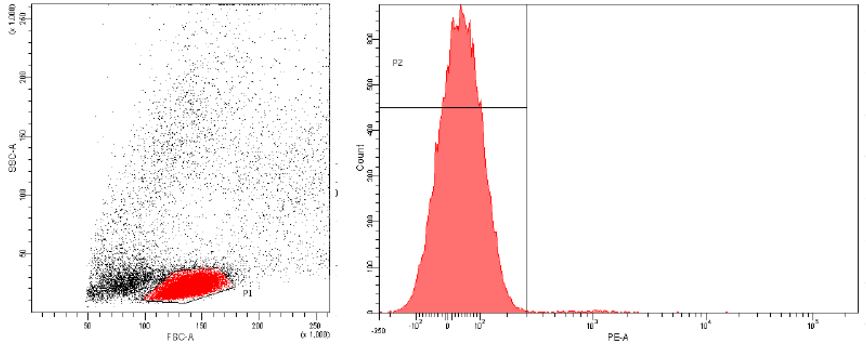
- Assay Configuration
  - Autologous unstimulated PBMCs
  - Autologous unstimulated PBMCs + Tregs
    - Different ratios of responders: Tregs
  - CD3/CD28 stimulated PBMCs
  - CD3/CD28 stimulated PBMCs + Tregs
    - Different ratios of responders: Tregs
  - Tregs alone
  - Autologous unstimulated PBMCs (for instrument setup)

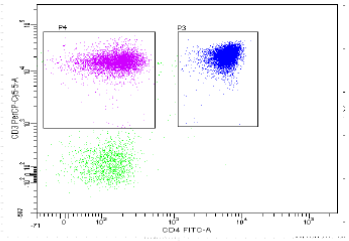
BD FastImmune Human Regulatory T Cell Function Kit

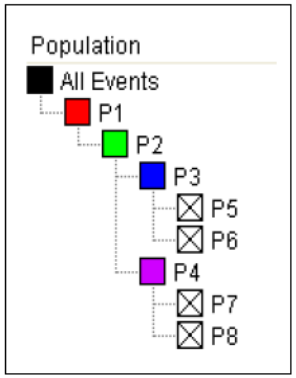
- Assay overview
  - Set up cell mixtures.
  - Activate cells with CD3/CD28 beads in the presence of CD154.
  - Incubate for 7 h in the presence of CD154.
  - Harvest and stain with
    - CD4/CD3/CD25 cocktail
    - CD69 (Can be omitted if using 4-color instrument)
  - Analyze on a flow cytometer.

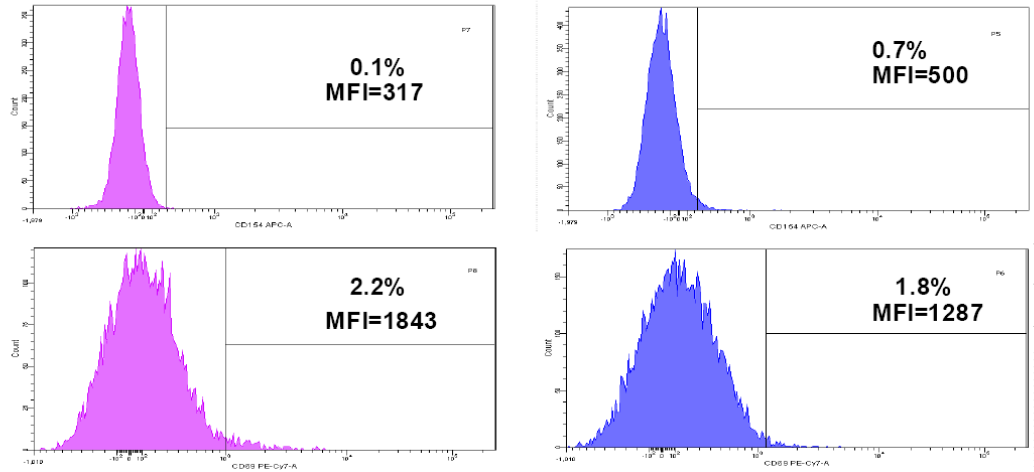
BD FastImmune Human Regulatory T Cell Function Kit

- Set gates using
  - Unstimulated and unstained PBMCs
  - Unstimulated PBMCs





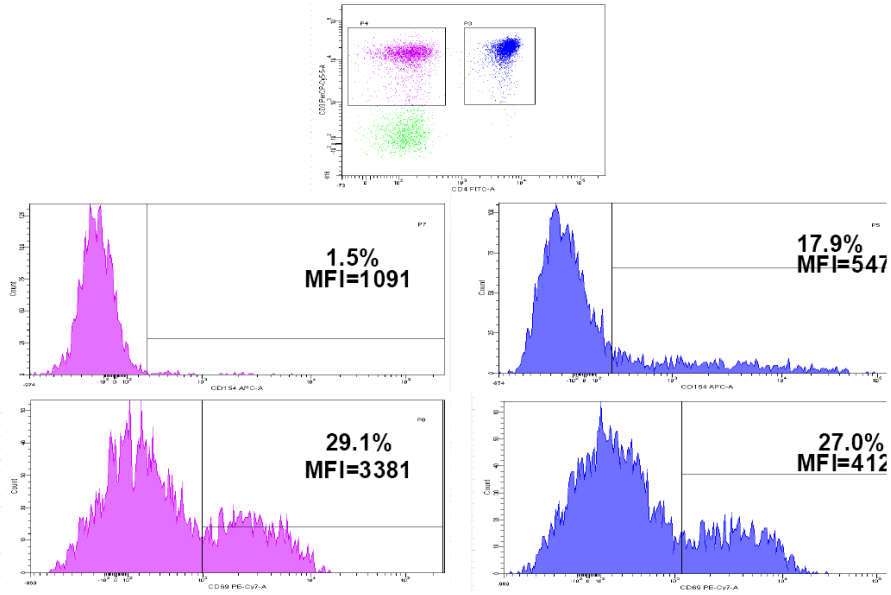




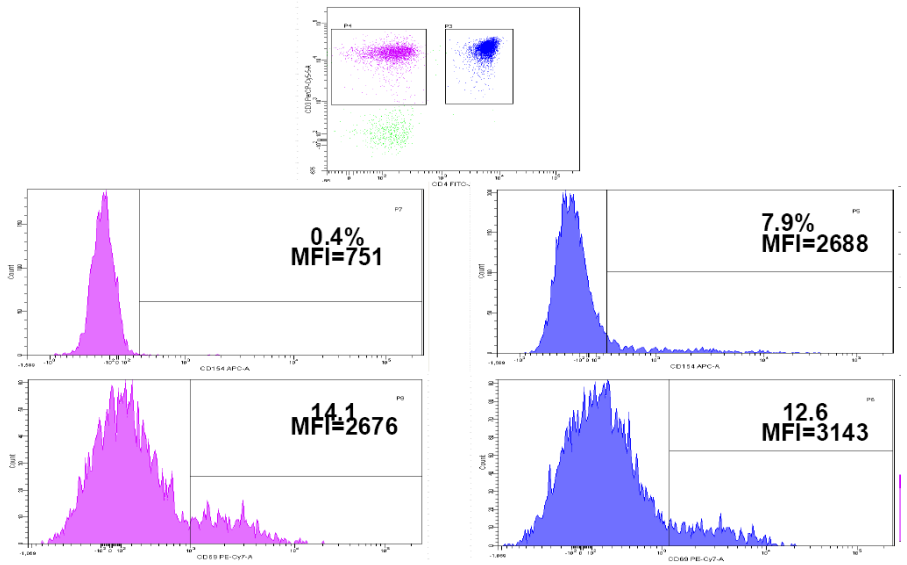
BD FastImmune Human Regulatory T Cell Function Kit

- Evaluate CD154 and CD69 responses

Stimulated PBMCs



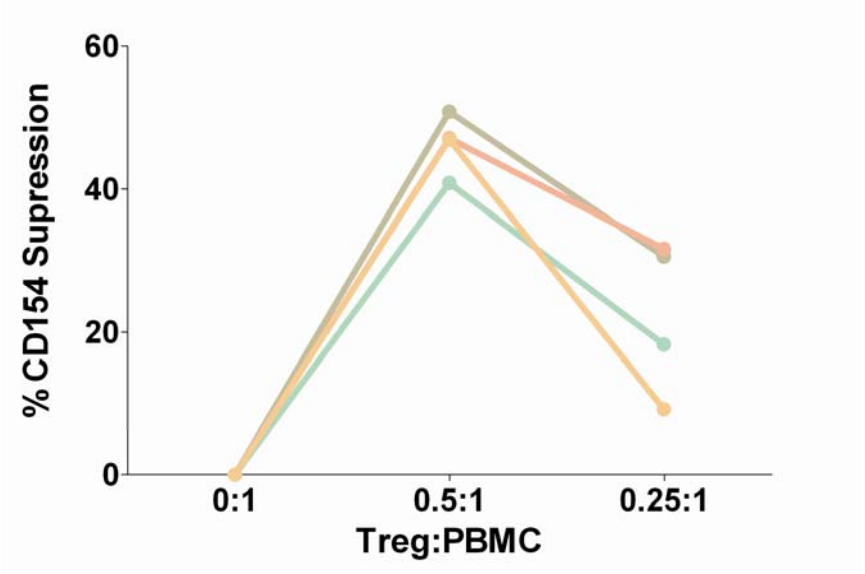
Stimulated PBMCs + Tregs

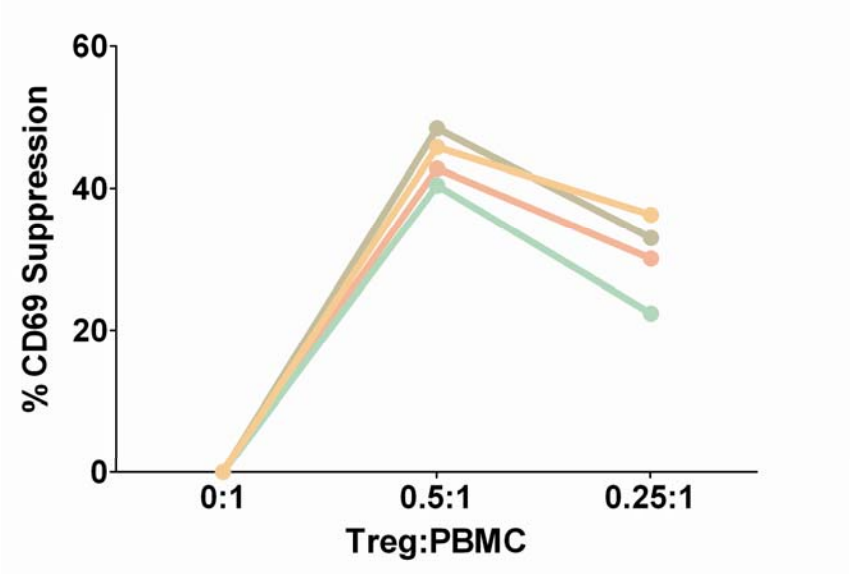


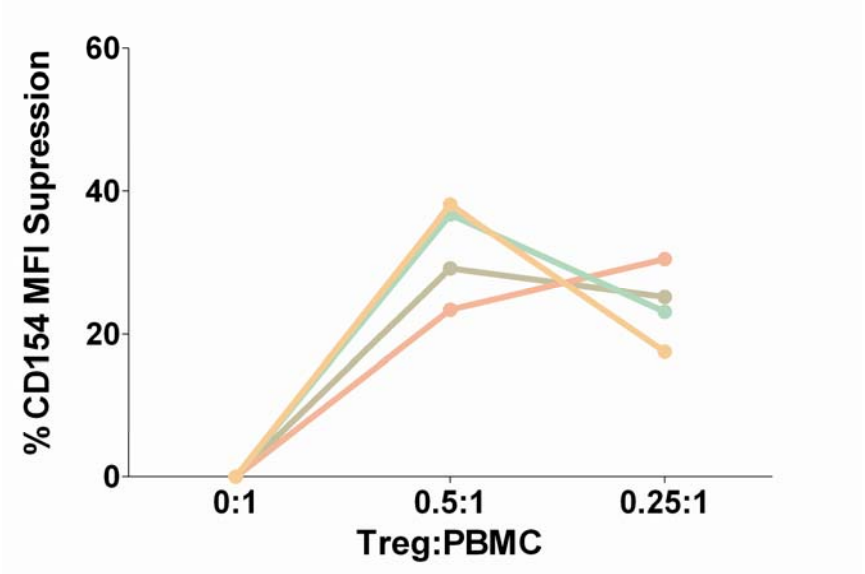
$$\text{\% suppression of marker frequency} = 100 - \frac{\text{\% positive in presence of Tregs}}{\text{\% positive in absence of Tregs}} \times 100$$

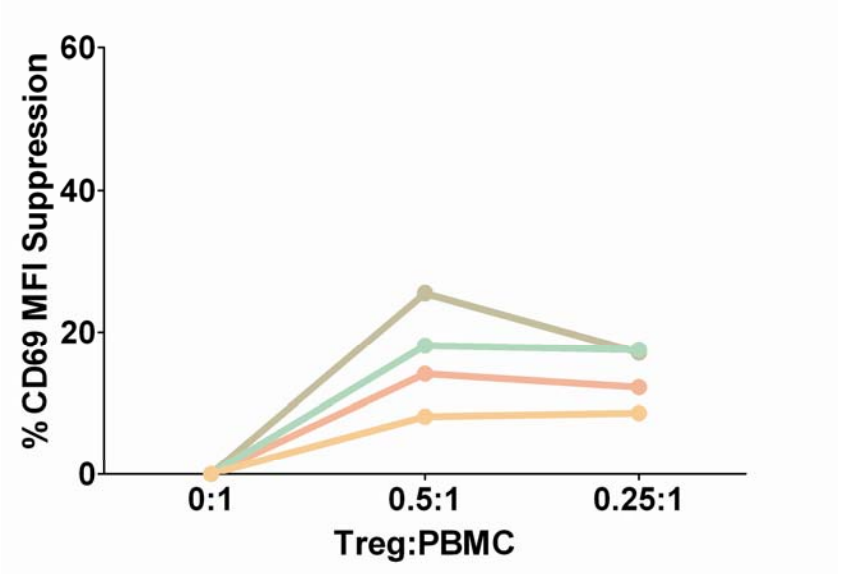
$$\text{\% suppression of marker expression level} = 100 - \frac{\text{MFI in presence of Tregs}}{\text{MFI in absence of Tregs}} \times 100$$

BD FastImmune Human Regulatory T Cell Function Kit









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Conclusions

- BD Regulatory T Cell Sorting Kit
  - $CD45RA^+$ and $CD45RA^-$ Tregs can be isolated from Human PBMCs.
    - High purity
    - High viability
    - High $\%FoxP3^+$
    - Cultured 13–14 days ($CD45RA^+$ 4 donors)
- BD FastImmune Human Regulatory T Cell Function Kit
  - Cultured Tregs are suppressive.

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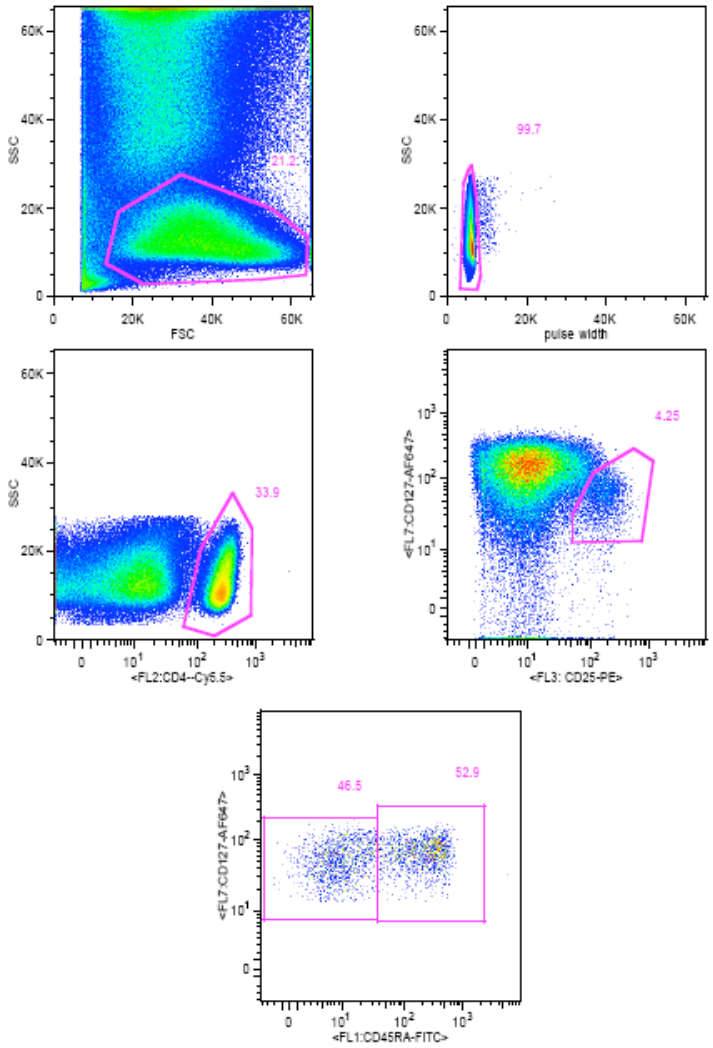
BD FACSAria III Flow Cytometer

- Key Benefits
  - Easy operation
  - Streamlined workflow
  - Experiment-to-experiment and operator-to-operator reproducibility
  - High-performance sorting and multicolor analysis
  - Lower total cost of ownership
  - Designed with Biosafety in mind
- Supporting features
  - Cuvette-based high-speed sorter
  - Alignment-free optical system
  - High-efficiency collection optics
  - Self-supporting fluidics system
  - Digital electronics

BD Influx System

- Key benefits
  - An open, configurable platform, adaptable to the needs of a wide range of applications
  - High-performance steam-in-air sorting
  - Comprehensive control of instrument allowing precision application setup and self-service
  - Controlled sorting environment
  - Elimination of sort to sort contamination
  - Amenable to large cell sorting and bioprocessing
- Supporting features
  - Independent laser steering and focusing
  - Integrated HEPA-filtered enclosure with a small footprint
  - Access to optics and fluidic controls
  - Exchangeable fluidics
  - Small particle option
  - Larger size nozzles

BD Influx – Human Regulatory T Cell Sorting Kit



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Practical Tips for Sorting

- Setting expectations
  - How many cells do you want from a sort?
  - Understand your process.
    - Recovery
    - Purity
    - Sampling
  - Treg example
    - 2% of PBMCs are $CD4^+CD25^+FoxP3^+$ lymphs
    - Percentage recoveries 45–100%

    So….

    To recover 1 x 10^6 Tregs, start with 1 x 10^8 PBMCs.
    - There are ~1 x 10^6 PBMCs in 1 mL of whole blood.
    - Might be appropriate to use buffy coats or apheresis packs.

Practical Tips for Sorting, continued

- Setting up your BD cell sorter
  - Consult the User's Guide.
  - QC your instrument.

The BD™ Cytometer Setup and Tracking (CS&T) is a fully automated system that provides instrument characterization, tracking, and quality control.

  - Use sorting templates provided.
    - Area scaling
    - Doublet discrimination
  - Check application settings.
    - Appropriate for dim and bright signal detection

BD FACS Diva™ software has templates for sorting and application setup.

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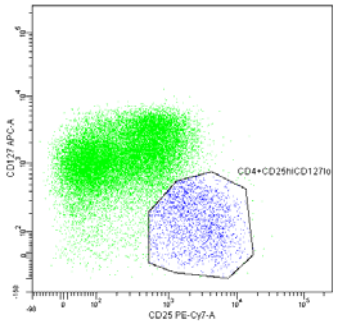
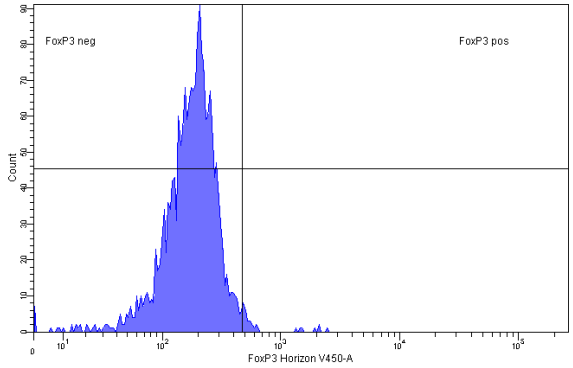
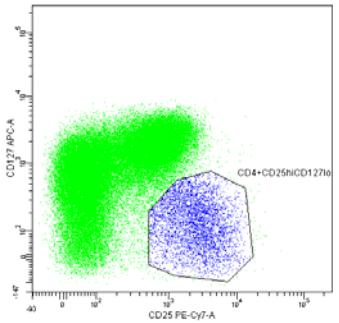
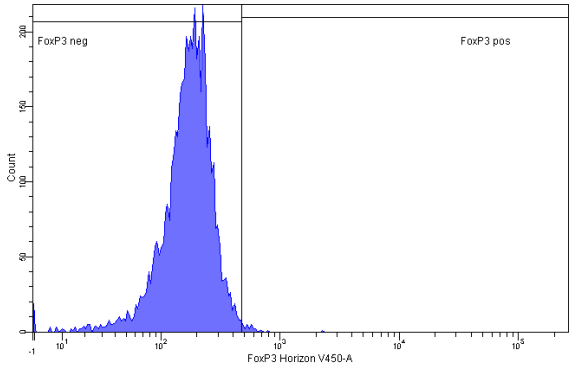
Practical Tips for Sorting, continued

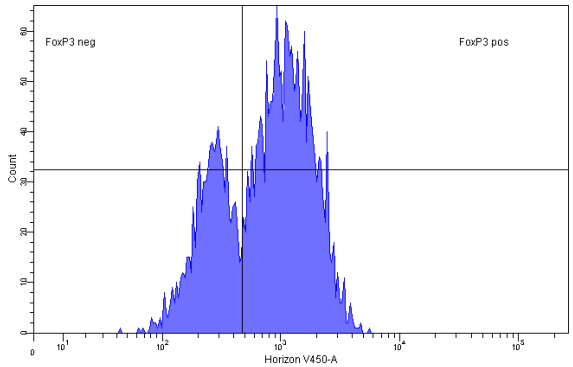
- Setting up your BD cell sorter
  - Use FMO controls

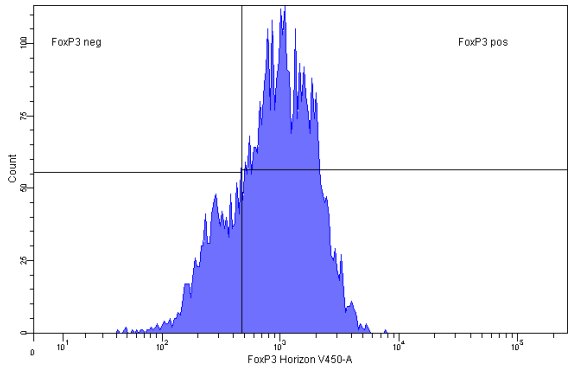










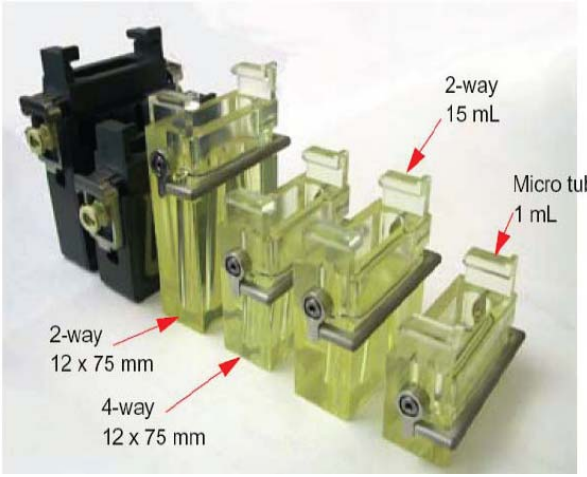


Practical Tips for Sorting, continued

- Setting up your BD cell sorter
  - Decontaminate your instrument.

    The Prepare for Aseptic Sort procedure on the BD FACSAria II and BD FACSAria III can eliminate bacteria and endotoxin contamination.

    The BD Influx has a fluidics kit that can be easily installed.
  - Choose appropriately sized collection tubes.
    - 15 mL
    - 12 x 75 mm (5 mL)
    - Microtubes (1 mL)
  - Align the sort stream carefully.
    - Cells go directly into liquid.
    - Do not hit sides of a tube.



Practical Tips for Sorting, continued

- Setting up your BD cell sorter
  - Choose the correct sort precision mode.
    - Purity mode results in very high purity at the expense of recovery and yield.
    - Yield mode results in high recovery and yield at the expense of purity.
  - Choose an appropriate event rate.
    - Sorting is optimized at lower flow rates.
    - An event rate that is too high will reduce yield

Practical Tips for Sorting, continued

- General tips on cell handling
  - Temperature fluctuations can affect
    - Viability
    - Recovery

The BD FACSAria II and BD FACSAria III can chill the

- Loading chamber
- Collection sort block

  - Serum and/or medium
    - Do not exceed 2% serum
    - Sort cells onto a liquid cushion

BD

Practical Tips for Sorting, continued

- General tips on cell handling
  - pH
    - Avoid bicarbonate-based buffers and media for sorting.
    - HEPES (up to 25 mM) will limit pH fluctuations.
  - Cell sedimentation can result in
    - Low event rates
    - Aggregate formation
    - Clogging

The BD FACSAria II and BD FACSAria III have a variable sample agitation feature.

Cells can be filtered using BD Falcon™ strainers.

BD

Practical Tips for Sorting, continued

- Tips for maximizing Treg viability
  - Minimize cell handling and centrifugation.
  - Minimize shear forces.
    - Avoid
      - Small gauge needles
      - Small pipet tips
      - Rapid pipetting
    - Try
      - Gentle pipetting
      - P1000 pipet tips

Practical Tips for Sorting, continued

- Tips for maximizing Treg viability
  - Minimize cell adherence.
    - Use polypropylene tubes.
    - Coat tubes with serum.

    The BD Falcon line offers many polypropylene tubes.
  - Use a cushion composed of
    - X-VIVO 15 medium
    - 10% Human AB serum
    - 0.4% acetylcysteine
  - Mix the sample.
    - Use the vriable sample agitation feature (BD FACSAria II and BD FACSAria III)
    - Periodically pause the sort and invert the sample tube.

Practical Tips for Sorting, continued

- Post-sort analysis
  - Use a pre-sort sample to set up the instrument.
  - Use FMO controls as appropriate.
  - Set the acquisition counter on a cell marker.
    - For Tregs, use $CD4^+$ events.
  - Resuspend cells in phenol red-free buffer.
  - Adjust the gate slightly, if required.

Practical Tips for Sorting, continued

- Post-sort culture of Tregs
  - Decontaminate the cytometer prior to sorting.

The Prepare for Aseptic Sort procedure on the BD FACSAria II and BD FACSAria III can eliminate bacteria and endotoxin contamination.

  - Limit post-sort handling.
    - Perform a single centrifugation.
    - Rest Tregs overnight prior to functional assays .

Overview

- Introduction to Tregs
- Reagents
  - Individual antibodies
  - Cocktails
  - BD Pharmingen Human Regulatory Cell Sorting Kit
  - BD FastImmune Human Regulatory T Cell Function Kit
- Instrumentation
  - BD FACSAria flow cytometers
  - BD Influx system
- Practical tips
  - Setting expectations
  - Setting up the BD FACSAria II and BD FACSAria III
  - Handling cells
  - Maximizing Treg viability
  - Performing post-sort analysis
  - Performing post-sort culture

Additional Information

- Application Notes
  - Human Regulatory T-Cell Isolation and Measurement of Function
  - Decontamination of the BD FACSAria II System Using the Prepare for Aseptic Sort Procedure
  - Reduction in Endotoxin Levels After Performing the Prepare for Aseptic Sort Procedure on the BD FACSAria II Flow Cytometer
- www.bdbiosciences.com

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For more detailed information on some of the experiments discussed in this presentation, please see our application note titled "Human Regulatory T-Cell Isolation and Measurement of Function."

Thank you.

BD